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MUTATION INDUCTION AT MULTIPLE GENE LOCI IN CHINESE HAMSTER OVARY CELLS: COMPARISONS OF BENZO(A)PYRENE METABOLISM BY ORGAN HOMOGENATES AND INTACT CELLS

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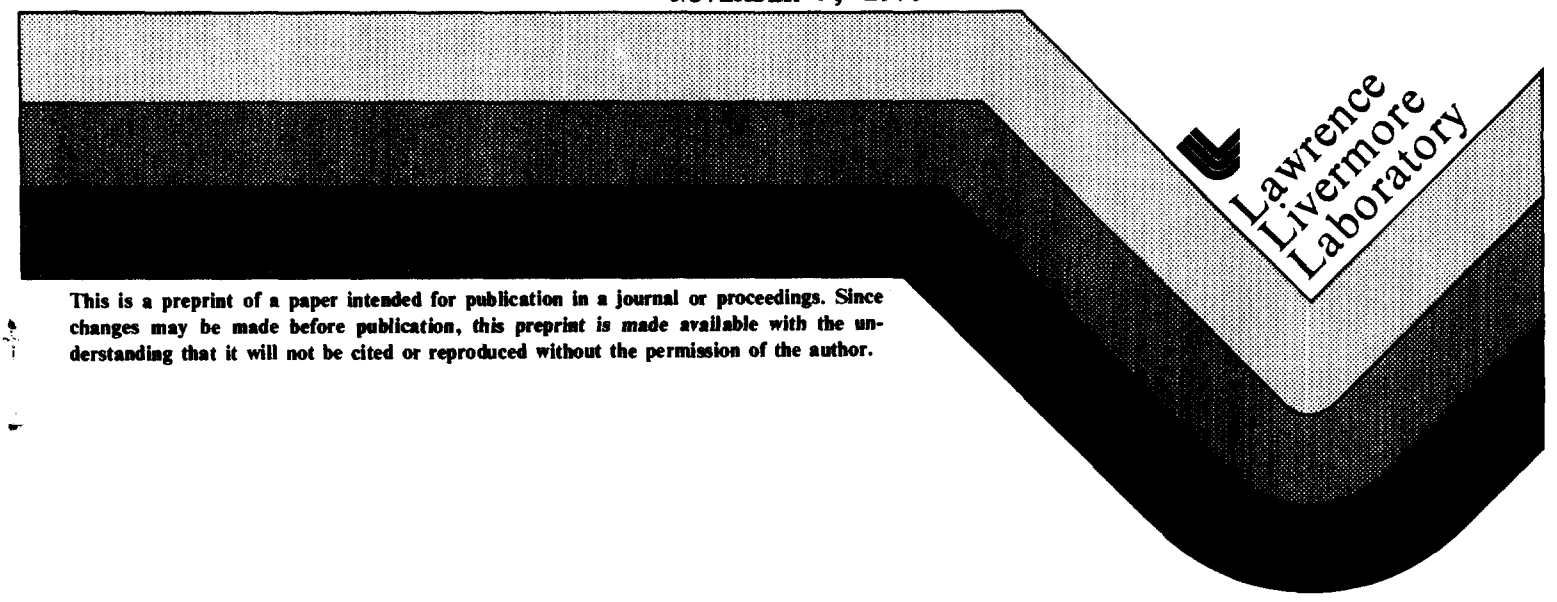

Mutation Induction at Multiple Gene Loci in Chinese Hamster Ovary Cells: Comparisons of Benzo(a)pyrene Metabolism by Organ Homogenates and Intact Cells.

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Running Title: Induced Mutation and B(a)P Metabolism

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Short-term, in vitro tests are gaining acceptance as a rapid means to assess the mutagenic and carcinogenic potential of chemicals found in the environment (3,19,21,26,27). Many of these chemicals require metabolic activation to electrophilic forms for mutagenic activity. Current mutagenesis assays rely on organ homogenates or intact cultured cells for metabolic activation. A key question is whether these activation systems duplicate, or even approximate, the response in the intact animal. The mutagenesis assay reported here seems to be a sensitive indicator of genetic damage in mammalian DNA (2). We have used four independent gene loci in our study. Because genetic loci may differ both in sensitivity and mechanism for mutational damage, the simultaneous use of several gene loci may be more effective in detecting potentially mutagenic agents or mixtures of compounds. Comparing the observed mutation induction with metabolite profiles produced under different conditions of in vitro metabolic activation will provide a data base leading to experiments more directly relevant to the complex in vivo bioactivation and detoxification processes.

Abbreviations in this report include: α-MEM, alpha minimum essential Eagle medium; AHH, aryl hydrocarbon hydroxylase; aprt, gene coding for adenine phosphoribosyltransferase, EC 2.4.2.7; ATPase, gene coding for NA^+ and K^+ activated, Mg^{++} dependent ATPase, EC 3.6.1.3; 7,8 diol, 7,8 dihydroxy-7,8 dihydrobenzo(a)pyrene; 9,10 diol, 9,10-dihydroxy-9,10 dihydrobenzo(a)pyrene; 4,5 diol, 4,5 dihydroxy-4,5 dihydrobenzo(a)pyrene; hgprt, gene coding for hypoxanthine-guanine phosphoribosyltransferase, EC 2.4.2.8; HPLC, high performance liquid chromotography; 3-OH, 3-hydroxybenzo(a) pyrene; 9-OH, 9-hydroxybenzo(a)pyrene; tk, gene coding for thymidine kinase, EC 2.7.1.21.

We have compared different methods of activating benzo(a)pyrene (B(a)P) in an in vitro mammalian mutagenesis assay (1,2). Metabolites of B(a)P are known to be toxic, mutagenic, and carcinogenic (4,11,13,14,16,20,32,33,34). The parent hydrocarbon is ubiquitous in the environment and is particularly released by processes associated with energy production, conversion, and utilization (5,9). Benzo(a)pyrene is metabolized by microsomal mixed-function oxidases, epoxide hydratase, and a variety of conjugases to form phenols, quinones, dihydrodiols, epoxides, and conjugates of the oxygenated metabolites (10,29,31,35). The 9,10-epoxides are believed to be the major mutagenic and carcinogenic metabolites of B(a)P (8,33).

In this report, mutations induced by B(a)P are quantified at four gene loci in Chinese hamster ovary (CHO) cells: aprt, hgprt, tk, and ATPase. The mutants were selected by their resistance to 8-azaadenine (AA), 6-thioguanine (TG), 5-fluorodeoxyuridine (FUdR), or ouabain (OUA). We compare activation by liver homogenates (S9) and liver microsomes from rats induced with Aroclor 1254, Syrian hamster embryo (SHE) cells, and kidney microsomes from male, female, and testosterone-treated female C3H/HeJ mice. Finally, a comparison is made between profiles of B(a)P metabolites produced by the different activation systems and the observed mammalian mutagenesis data.

MATERIALS AND METHODS

PREPARATION OF ORGAN HOMOGENATES AND SHE CELLS

For the Aroclor-induced rat-liver material, eight-week-old outbred male rats (Simonsen albino, Sprague-Dawley derived) were injected i.p. with

Aroclor 1254 (200 mg/ml in corn oil) at 500 mg/kg body weight. Five days later, rats were sacrificed by arterial bleeding after anesthesia in a CO_2 atmosphere. Livers were removed under sterile conditions, washed with cold phosphate buffered saline (PBS), and homogenized in 3 mls PBS/g wet liver weight with a Polytron homogenizer (Brinkmann Instruments) for 1 minute at setting 4. The resulting homogenate was centrifuged at 9000 x g for 15 min at 4°C. The supernatant (S9) was frozen and stored at -80°C. To prepare microsomes, the S9 was immediately centrifuged at 100,000 x g for 1 h. The pellet was gently homogenized, resuspended in sterile 10% glycerol in PBS, and stored at -80°C. The mouse kidney microsomes were prepared from 8- to 10- week-old C3H/HeJ male and female mice. Testosterone pellets (30 mg) (kindly supplied by Dr. Richard Swank, Roswell Park Memorial Institute) were implanted subcutaneously in the backs of selected females 8 days before sacrifice. All protein concentrations were determined by the method of Lowry et al., (17).

Primary Syrian hamster embryo (SHE) cells were obtained by a modification of R.J. Pienta's protocol (personal communication). Adult female hamsters were sacrificed and embryos were removed and decapitated. Embryonic tissues were dispersed into single cells by successive treatments with 0.05% trypsin at room temperature. Dispersed cells were incubated in flasks at 37°C for 24 h, the debris was decanted, and the mixed cell population trypsinized and resuspended in α-MEM culture medium containing 10% glycerol and 10% fetal calf serum. Samples were frozen in liquid nitrogen for long term storage.

CELL CULTURE AND MUTAGENESIS ASSAYS

The parent cells for these studies were Chinese hamster ovary (CHO) cells obtained from Dr. W.C. Dewey. A subline, CHO-AT3-2, was selected and characterized biochemically as heterozygous for both the aprt and tk loci (1), permitting single-step selection of mutants resistant to AA (aprt$^{-/-}$), TG (hgprt^{-}), FUdR (tk$^{-/-}$), or OUA (altered ATPase) (2). Approximately 18 h before treatment with promutagen, CHO-AT3-2 cells were plated at 1 to 2 x 10^6 cells per T25 flask. Cells were exposed for 2 h to the promutagen in α-MEM (serum-free) containing the activation system and the following co-factors: NADPH, 0.37 mM; NADH, 0.93 mM; NADP, 0.87 mM; glucose-6-phosphate, 6.57 mM. For activation by SHE cells, the CHO-AT3-2 cells were coincubated with lethally-irradiated SHE cells and the promutagen for 48 h at a CHO:SHE ratio of 1:1 which increased to approximately 8:1 by the end of the incubation. After either 2 h or 48 h of exposure, approximately 1 to 2 x 10^6 CHO-AT3-2 cells were transferred into monolayer culture for expression of mutant (drug-resistant) phenotypes. After 3 days incubation, 3 to 5 x 10^6 cells were placed in suspension culture for continued phenotypic expression and subsequent replating for mutant selection. Plating efficiencies ranged from 0.65 to 0.85 (data not shown). The observed mutant frequency is the ratio of mutant colonies per dish to the number of viable cells plated per dish. The induced mutant frequency is the difference between the observed mutant frequency of treated cultures and the observed spontaneous mutant frequency of untreated culture (controls).

ASSAY OF B(a)P METABOLITES

For HPLC analysis, all activation systems plus cofactors were added to α-MEM culture medium and incubated at 37°C for 15 min, except for

kidney microsomes which were incubated for 30 min. To each reaction, 5.0 μCi of randomly-labeled [^{3}H]B(a)P (diluted to a specific activity of 0.14 Ci/mmole, Amersham-Searle) was added. For analysis of SHE metabolism, the cells were incubated with B(a)P for 48 hours, scraped from the flask with a rubber policeman, and suspended in the culture medium for extraction. To terminate the reaction, 1 ml of cold acetone was added per ml of α-MEM medium. The acetone-α-MEM mixture was extracted with ethyl acetate (2 ml/ml medium), and the organic phase was removed, dried under nitrogen and resuspended in methanol (200 μl). Twenty-five μl of this solution was used for HPLC analysis on a Hewlett Packard 1084B equipped with an RP-18 column. Metabolites were separated using a methanol-water gradient (40:60 to 90:10 in 30 min) at a flow rate of 4 ml/min. Fractions were collected every 0.5 min for 35 min. Peaks were identified by comparison of retention times to standards provided by IIT Research Institute, Chicago, Illinois, through the National Cancer Institute Chemical Repository. Cytochrome P-450 concentration and AHH activity were determined by the methods of Omura and Sata (25) and Nebert and Gelboin (23), respectively. The demethylase activity was determined according to Frantz and Malling (7) with measurements of formaldehyde production as described by Nash (22).

RESULTS

METABOLIC ACTIVATION OF B(a)P: CELL TOXICITY

Figure 1 shows the relative cell survival, or the fraction of surviving CHO-AT3-2 cells after B(a)P treatment, decreased with the concentration of activating protein (mg/ml). With 10 μg/ml of B(a)P, cell toxicity with activation by rat liver S9 and microsomes increased up to 1.50 and 0.75 mg/ml respectively. No significant cell toxicity was observed with kidney microsomes from male mice (0.5 to 1.0 mg/ml) at B(a)P concentrations ranging from 20 to 150 μg/ml. Slight toxicity was seen at 100 μg/ml B(a)P with kidney microsomes from untreated and testosterone-treated female mice. After 48 hours, significant toxicity ($\geq$20% relative cell survival) was observed for CHO-AT3-2 cells coincubated with SHE cells at B(a)P concentrations ranging from 2 to 10 μg/ml (data not shown).

MULTIPLE MARKER MUTAGENESIS ASSAY: DRUG-RESISTANCE MARKERS

Figure 2 shows the increase in frequency of the induced mutations as a function of relative cell survival after treatment with B(a)P, activated by several metabolic activation systems. For comparison, the response to ethyl methanesulfonate (EMS) and dimethylnitrosamine (DMN) activated by rat liver S9 is given in the legend to Fig. 2, expressed as a function of relative cell survival after mutagen treatment. The frequency of mutations induced after activation by rat liver microsomes was similar to that after SHE activation. Rat liver S9 produced metabolites that were apparently toxic but only slightly mutagenic to CHO-AT3-2 cells. Minimal mutagenesis was observed from B(a)P activated by mouse kidney microsomes.

The B(a)P activated by kidney microsomes from male mice was neither toxic nor mutagenic; preparations from females produced some toxic responses but no significant mutation. Microsomes from females treated with testosterone slightly increased the spontaneous mutation frequencies at two out of the four loci (OUA^R and $FUdR^r$). No additional toxicity or mutagenicity was observed at B(a)P concentrations up to 150 μg/ml.

In contrast to minimal B(a)P-induced mutagenesis after activation by kidney microsomes is the ability of C3H/HeJ mouse kidney microsomes to metabolize DMN to mutagenic intermediates. At $\geq$10% survival, preparations from male kidney yielded DMN-induced mutation frequencies approximately 3- to 22-fold higher at all four loci than those of controls or with microsomes from female mice; kidney microsomes from testosterone-treated females increased DMN-induced mutations from 4- to 20 fold greater than controls and microsomes from untreated females (Fig. 3). Differences between B(a)P- and DMN-induced mutation with mouse kidney microsomal activation can be seen in Table 1. Kidney microsomes were compared for a number of metabolic properties. The cytochrome P-450 content was eight fold higher in male kidney microsomes than that in female preparations; AHH activity did not differ in males and females. Implanting testosterone pellets in females markedly increased both cytochrome P-450 content and DMN demethylase activity, but did not affect AHH activity.

B(a)P METABOLITE PROFILES

Figure 4 shows the B(a)P metabolite profile obtained with 0.25 mg/ml of rat liver microsomal protein under culture conditions. Quantitation of metabolite profiles obtained with varying concentrations of rat liver S9 or microsomes or intact SHE cells is given in Table 2. Total

B(a)P metabolism with microsomes increased with up to 0.75 mg/ml protein, then slowly decreased. Consistent levels of 3-OH, 7,8 diol, and 9,10 diol with increasing amounts of activating system indicate that steady state levels of these compounds are formed under conditions of increasing total metabolism. In contrast, the 4,5 diol levels decrease. Tetrol or conjugate levels increase with microsomal protein concentration up to 0.75 mg/ml, implying an increase in transient levels of diol epoxides which are too reactive to be measured directly.

With rat liver S9, the total metabolism increased with increasing protein. At similar protein concentrations (0.75 mg/ml), metabolite levels are generally either similar to or higher than those measured with microsomal activation. Production of both 3-OH and 9-OH by S9 protein increases beyond the levels produced by microsomes.

In contrast to rat liver metabolism, intact SHE cells produced little or no tetrols. Amounts of water-soluble metabolites are relatively high, which may result from formation of water-soluble B(a)P conjugates (8) during the 48 hr incubation period. The major ethyl-acetate-extractable metabolite formed is the 7,8 dihydrodiol. This intermediate is the proximal precursor to the very mutagenic 7,8 dihydrodiol-9,10 epoxide.

Kidney microsomes from C3H/HeJ mice that were not induced with Aroclor were unable to generate sufficient quantities of mutagenic metabolites of B(a)P to yield significant mutagenesis. As seen in Table 1, total metabolite production was only 11 to 14%, and specific measurement of the fluorescent 3-OH product (AHH activity) was less than 5 pmol/mg protein·min, in contrast to 5,000 pmol 3-OH B(a)P from Aroclor-induced rat liver microsomes (data not shown). However, cytochrome P-450 levels in

male and testosterone-treated female mice are sufficient to demethylate DMN in significant quantity (Table 1). This level results in dose-dependent increases in mutation frequency at all four gene loci (Fig. 3) (Felton and Carver, in preparation).

DISCUSSION AND CONCLUSIONS

In brief, the monooxygenase system is responsible for incorporation of one atom of molecular oxygen into an aromatic substrate (in this report B(a)P) resulting in an arene oxide (epoxide) intermediate, see ref. 31 for review. This reactive intermediate can upon spontaneous rearrangement form a phenol, be catalyzed by epoxide hydratase to a dihydrodiol or by glutathione transferase to a glutathione conjugate, or become covalently bound to nucleic acids resulting in a mutagenic event. The phenols can be further conjugated to even more polar forms by UDP glucuronyl transferase or an appropriate sulfatase. The epoxide and dihydrodiol can be further metabolized by the monooxygenase system to form the very mutagenic diol epoxides. Finally, these reactive intermediates can be hydrolyzed spontaneously or be converted to tetrols by the epoxide hydratase.

Although significant differences are observed in B(a)P-induced mutagenesis after activation by rat liver microsomes or S9, the measured metabolite profiles are very similar. This is somewhat surprising because at any given protein concentration there is approximately 4.6 times more cytochrome P-450 in the microsomes than S9. At comparable protein concentrations, the S-9 preparations produce higher levels of phenols (both 3-OH and 9-OH) than do the microsomes; these phenols may be more toxic than mutagenic to the CHO cells. This could account for the lower mutagenesis

seen in Figure 2 with increasing S9 concentrations. Alternatively, recovery of metabolized B(a)P decreases rapidly with increasing microsomal protein concentration, suggesting more covalent binding to macromolecules (including DNA), ultimately resulting in more mutations. The loss in recovery with increasing protein concentration could also be due to covalent binding to the increased concentration of soluble proteins present in the S9. However, Krahn and Heidelberger (15) reported significant B(a)P-induced mutagenesis in V79 cells after activation by rat liver S9 at 2 mg/ml total protein. Similar to the Selkirk metabolite study (28), these rats were induced with 3-methylcholanthrene. The metabolite profile from liver microsomes obtained from rats induced by 3-methylcholanthrene (30) is similar to that in Fig. 4, except that S9 from Aroclor-induced rat liver homogenates shows less 3-OH in the profile.

A good comparison of covalent binding or DNA adduct formation following metabolism by microsomes and S9 may lead to a better understanding of the marked differences between the two subcellular fractions. It is apparent that steady-state levels of metabolites (such as we have measured here) may not be a good indicator of mutagenic metabolism; exact measurement of interaction with the target may be more meaningful.

The metabolite profile obtained after SHE activation in our study differs in several aspects from data previously reported (18,29). In this study, the B(a)P concentration and reaction time were optimized for CHO mutagenesis, i.e., 10-fold higher concentration and 48 h incubation as opposed to 24 h. The data in Table 2 represent a combination of metabolites in cells and in the medium; both were potential sources of metabolites for CHO cells cocultivated with SHE cells. Most significant is the high level

of 7,8 diol and the low level of tetrols. In marked contrast to a previous report (18), our SHE activation yielded a larger fraction of 7,8 diol and 4,5 diol than of 9,10 diol forms and the 3-OH product exceeded the 9-OH form. The 7,8 diols, which are major metabolites, probably contributed heavily to SHE-activated B(a)P mutagenesis via 7,8 diol-9,10 epoxides. These diol epoxides may be more efficiently transported to the CHO target cells, with little or no accumulation of the diol epoxides and subsequent conversion to tetrols. Mutation results at the four gene loci were quantitatively similar to those from rat liver microsomes obtained after B(a)P activation, suggesting that both activation systems have the potential to produce significant levels of the mutagenic epoxide - SHE as evidenced by the 7,8 diol accumulation and microsomes by the presence of tetrols.

For two of the markers reported here (OUA^R and TG^r), the B(a)P-induced mutation measured in CHO cells after SHE activation can be compared to results in similar assays employing a metabolizing feeder layer combined with the cells used for selection of mutant phenotypes. Huberman and coworkers (11,12) used Syrian (golden) hamster cells with V79 hamster cells. Their data for OUA^R are similar to those shown in Fig. 2, with results for AG^r about 10-fold higher than our data for TG^r, consistent with the AG^r/TG^r ratio of ten observed in our CHO system (data not shown). Newbold and colleagues (24) also used V79 cells, with BHK cells for metabolism. Their reported increase in OUA^R mutants is about 20-fold higher than our results, and their data for AG^r are about 25-fold above the results shown in Fig. 2.

Kidney microsomes that have measurable cytochrome P-450 levels, metabolize DMN sufficiently to give dose-dependent mutagnesis (Fig. 3). The

same microsomes metabolize very little B(a)P (11 to 14%) and produce even less potentially mutagenic products, as reflected by minimal cell toxicity and very little mutagenesis (Fig. 2). Presumably the cytochrome P-450 in kidney from untreated female mice does not oxidize B(a)P efficiently, but does demethylate DMN efficiently.

In conclusion, we have compared profiles of B(a)P metabolites produced by different activation systems with the observed mutagenic activity at four gene loci in a mammalian mutagenesis system. Metabolite profiles for each activation system showed some striking similarities but some significant differences. Although profiles of SHE-activated B(a)P may indeed represent metabolism that is more relevant to the intact cell than to disrupted cell homogenates (29), induced mutation levels at four separate gene loci were very similar for B(a)P activated by rat liver microsomes or SHE. Thus, at least for B(a)P, dissimilar activation systems may produce similar steady-state levels of the mutagenic epoxide intermediates. However, the frequencies of B(a)P mutation with S9 activation were substantially lower. Kidney microsomes from male and testosterone-treated (but not from untreated) female mice efficiently metabolized DMN, but produced insignificant amounts of B(a)P metabolites; mutation data paralleled these results.

The usefulness of <u>in vitro</u> mammalian mutagenesis tests to screen potentially mutagenic chemicals will depend in part on the applicability of the assays in detecting mutagens and promutagens from a large variety of chemical classes. For activation of B(a)P, rat liver microsomal preparations were as efficient as feeder layers of intact cells. Whatever the reasons for the lower efficacy of rat liver S9, it is clear from these

studies that microsomal activation should be added to the usual protocol employing S9 activation of promutagens. This addition would enhance the ability of mutagenesis assays to detect mutagens that are metabolized by metabolic pathways similar to B(a)P.

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Reference to a company or product name does not imply approval or recommendation of the product by the University of California or the U.S. Department of Energy to the exclusion of others that may be suitable.

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TABLE 1. MOUSE KIDNEY METABOLISM:

COMPARISON OF B(a)P AND DMN

	Cyt. P_{450}[a]	DMN Demethylase[b]	AHH[c]	B(a)P Metabolism[d]
C3H ♂	0.39	2.9	4.0	11.0
C3H ♀	0.05	0.4	4.5	11.4
C3H ♀′ e	0.37	3.0	4.4	13.8

a nmol P-450/mg protein.

b nmol HCHO formed/mg protein·min.

c pmol 3-hydroxy B(a)P formed/mg protein·min.

d percent of counts not found in B(a)P peak.

e implanted with testosterone pellets.

TABLE 2. BENZO(a)PYRENE METABOLISM IN CULTURE

Activation System	Protein mg/ml	Benzo(a)pyrene Metabolite Profile (cpm x 10^{-3})								Unmetabolized B(a)P	Recovery[c] (%)
		Unident[a]	Tetrols, Conjug[b]	9,10 diol	4,5 diol	7,8 diol	9-OH	3-OH	Aqueous		
Rat liver microsomes	0.25	13.3	9.6	34.7	15.5	20.2	9.7	27.4	64	243	69
Rat liver microsomes	0.50	12.1	16.2	35.3	12.1	22.0	10.8	30.6	98	141	62
Rat liver microsomes	0.75	15.6	23.1	29.9	7.6	18.8	4.2	22.7	122	86	56
Rat liver microsomes	1.00	6.9	10.8	24.7	7.5	14.5	17.1	23.0	75	95	48
Rat liver microsomes	1.50	7.7	18.1	17.0	4.6	13.5	9.5	18.8	108	160	60
SHE	-	.01	.2	4.5	7.0	19.5	.01	9.0	215	220	85
Rat liver S9	0.125	3.1	4.2	11.9	9.1	8.7	1.0	14.1	49	493	75
Rat liver S9	0.250	8.3	5.8	18.7	9.6	17.5	6.5	23.4	67	446	77
Rat liver S9	0.50	8.1	11.9	37.2	15.0	26.2	15.0	40.2	95	255	65
Rat liver S9	0.75	13.8	13.0	37.6	14.7	29.5	18.5	46.9	122	180	62

[a] Unidentified polar metabolites.
[b] Presumed tetrols and sulfate conjugates.
[c] Percent Recovery equals total of organic metabolites, aqueous metabolites, and unmetabolized BP divided by radioactivity added to dish.

FIGURE LEGENDS

Figure 1. Relative cell survival of CHO-AT3-2 cells decreased with increasing concentrations of rat liver S9 (△) or microsomes (▲) or kidney microsomes from female mice untreated (●) or treated with testosterone (♦). Rat S9 and microsomes were incubated with 10 μg/ml B(a)P and mouse activation protein with 100 μg/ml.

Figure 2. The induced frequency of AA^r, TG^r, OUA^R, or $FUdR^r$ mutants increased with decreasing relative cell survival (fraction of surviving CHO-AT3-2 cells) after treatment with 10 μg/ml B(a)P [rat liver S9 (△), microsomes (▲), and SHE (■)] and 100 μg/ml B(a)P [mouse kidney microsomes (♦)]. The straight line fits of the curves for rat liver microsomes have the following slopes: AA^r, 4.2×10^{-4}; TG^r, 6.3×10^{-4}; OUA^R, 1.6×10^{-4}; FUdR, 2.1×10^{-2}. For comparison, the responses to EMS and DMN activated by rat liver S9 for each locus have the following slopes: AA^r, 3.3×10^{-3} and 4.8×10^{-4}; TG^r, 4.4×10^{-3} and 7.7×10^{-4}; OUA^R, 1.1×10^{-3} and 1.6×10^{-4}; $FUdR^r$, 1.4×10^{-2} and 1.9×10^{-2}.

Figure 3. Relationship of frequencies of AAr, TGr, OUAR, and FUdRr mutants with decreasing relative cell survival ($\bar{S}$) after treatment with 60 to 100 mM DMN and microsomes (open bars = 0.5 mg/ml protein and closed bars = 1.0 mg/ml) from male, female, or testosterone-treated female (♀′) C3H/HeJ mice. The observed, spontaneous mutation frequency at each locus is shown by the horizontal bar on the ordinate.

Figure 4. HPLC profile of Benzo(a)pyrene metabolites formed during a 15-min incubation of [^{3}H]B(a)P (0.14 Ci/mmol) with 0.25 mg/ml rat liver microsomal protein. Peaks are identified by correlation of retention times with known standards.

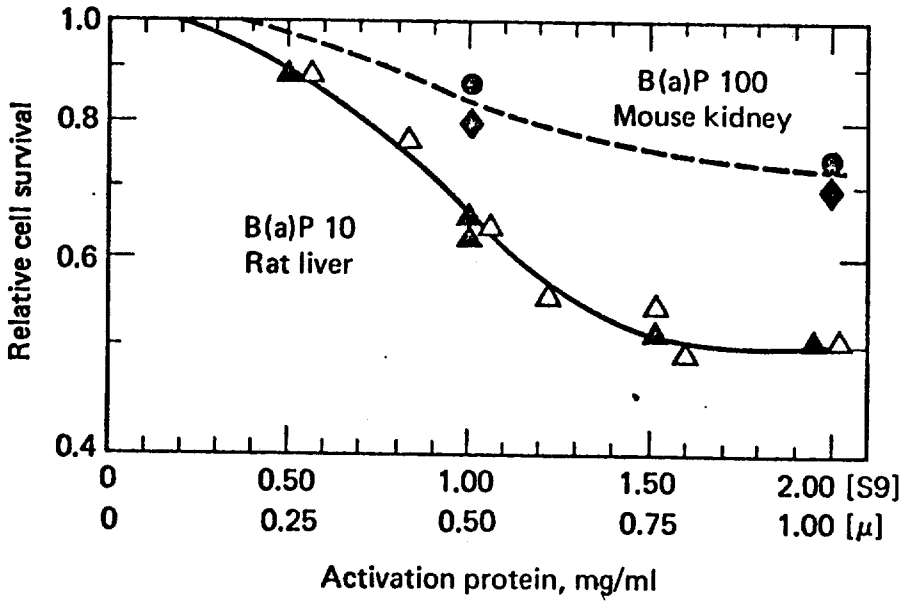

Figure 1
Carver, et al.

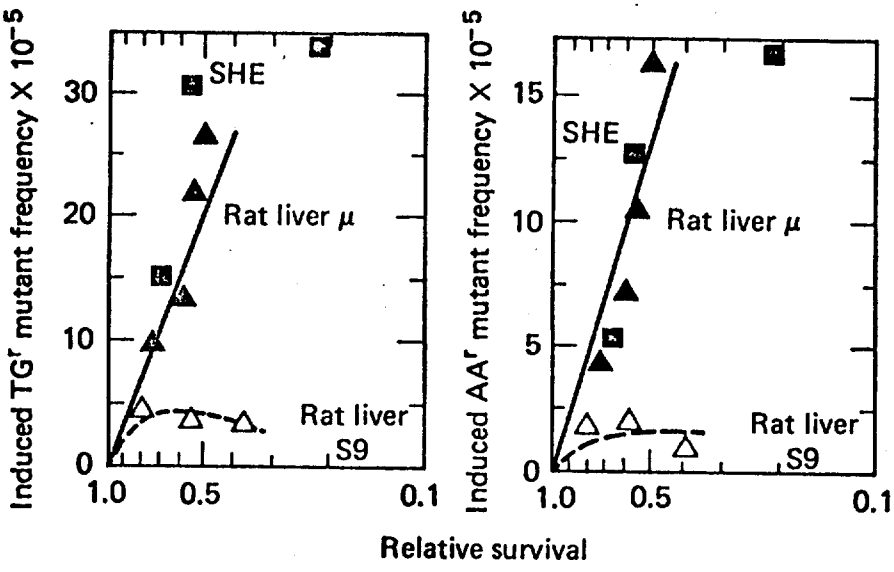

Figure 2a
Carver, et al.

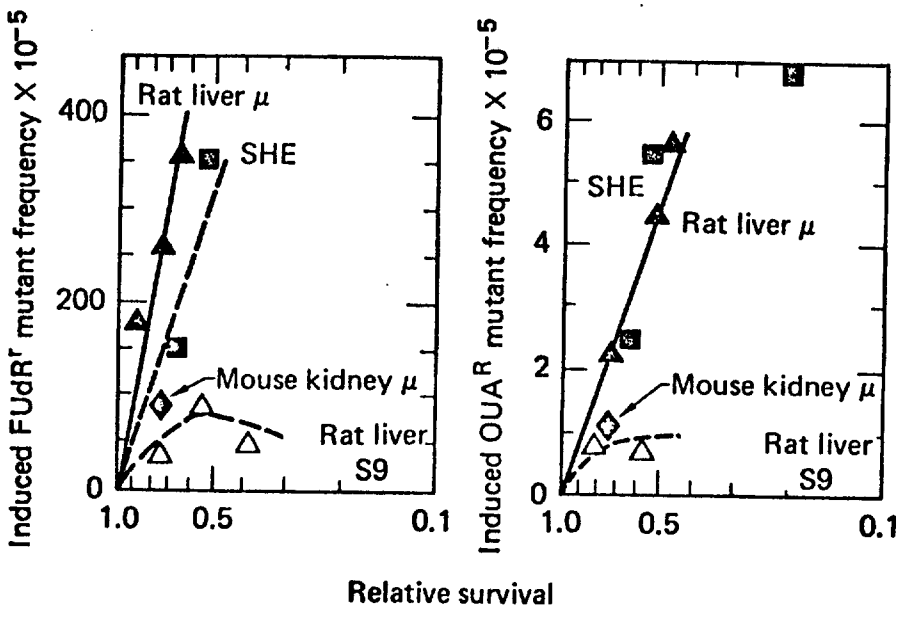

Figure 2b
Carver, et al.

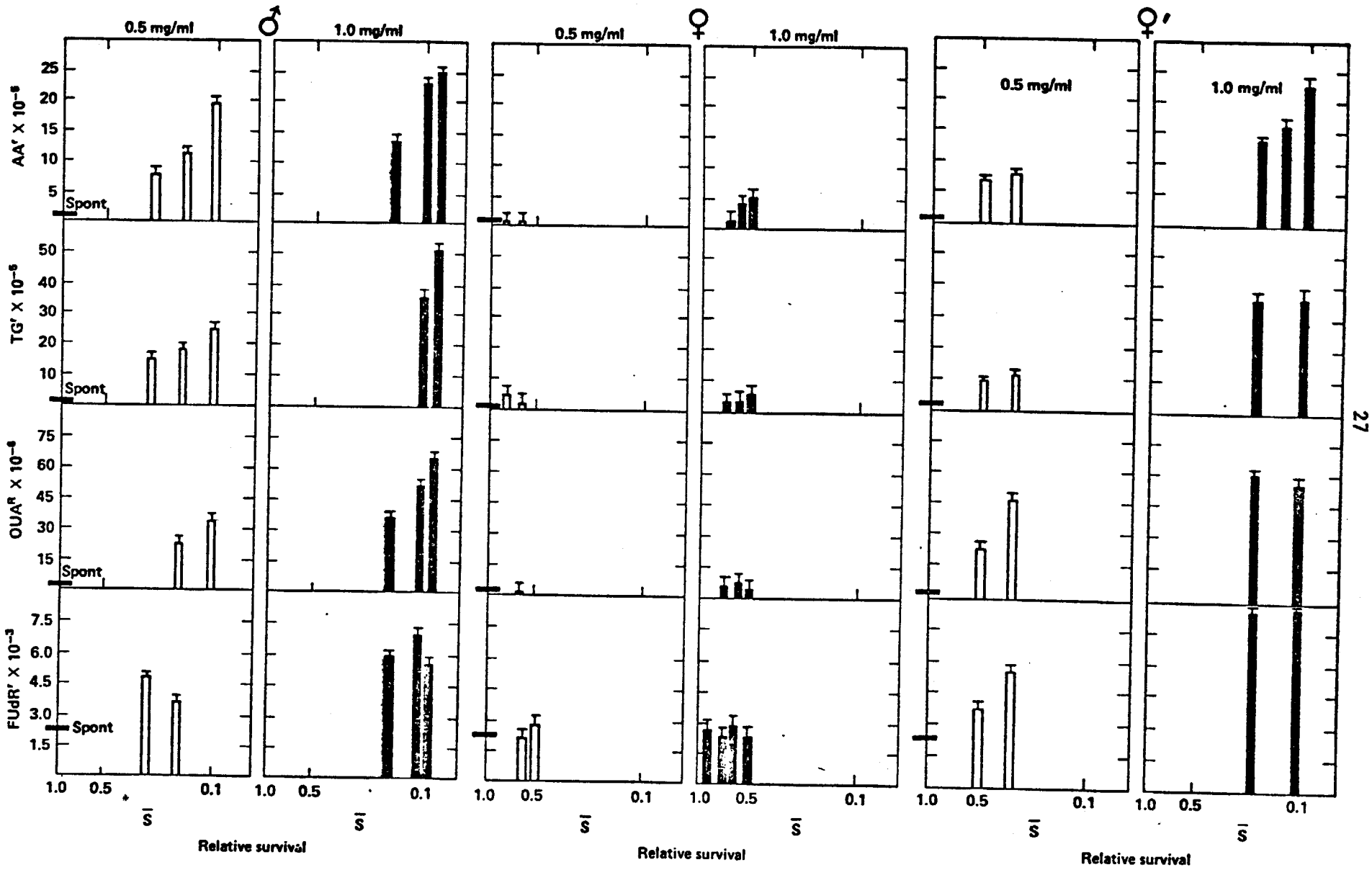

Figure 3
Carver, et al.

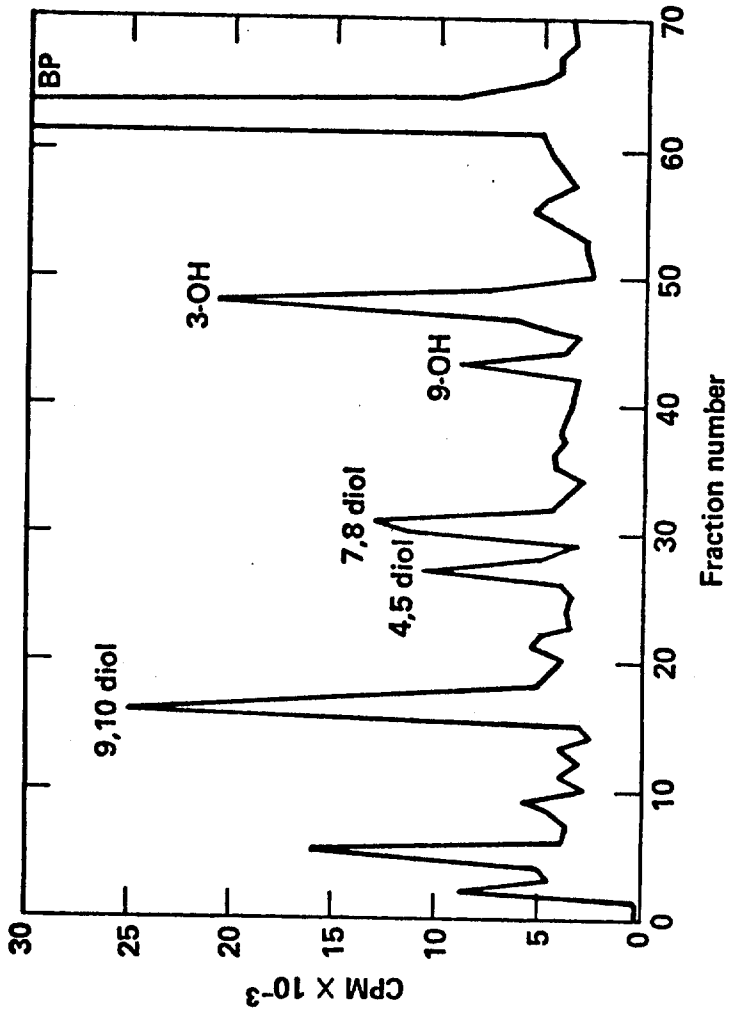

Figure 4
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